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5. Cellular Immunology, 1999 Apr 10, 193(1):48-58

DEFICIENT FAS-LIGAND EXPRESSION BY SYNOVIAL LYMPHOCYTES OF PATIENTS WITH RHEUMATOID ARTHRITIS. M.J. Cantwell¹, I. Hua², N.J. Zvaifler³, and L.J. Kings. Divisions of Hematology/Oncology and Rheumatology; UCSD School of Medicine, La Jolla, CA.

Mice that have defective expression and/or function of Fas (CD95) or its ligand develop lymphoproliferative and/or autoimmune disorders resulting from dysregulated lymphocyte activation. We assessed whether dysregulated expression of Fas (CD95) or its ligand also was associated with human autoimmune disease. For this we examined the lymphocytes in the blood and affected joints of patients with rheumatoid arthritis (RA), an autoimmune disease characterized by infiltration of peripheral joints with "activated" lymphocytes. We found that nearly all the lymphocytes in the synovial fluid or synovial tissue of diseased joints expressed high levels of CD95. In contrast, the blood lymphocytes of patients with RA did not express significantly higher levels of CD95 than lymphocytes of age-matched normal control subjects. In addition, we found that both synovial fluid and synovial-tissue lymphocytes were rapidly induced to undergo apoptosis upon *in vitro* culture with the anti-CD95 antibody, CH-11. However, we found that RA synovial fluid did not contain soluble CD95 or other specific inhibitors capable of inhibiting apoptosis induced by naive human Fas-ligand expressed by transfected mouse fibroblasts. Instead, we detected little or no expression of Fas-ligand by immunohistochemistry or sensitive RT-PCR in synovial-fluid mononuclear cells or synovial-tissue cells of patients with RA. The lack of Fas-ligand expression could not be explained by an inherent defect of RA synovial T cells in their ability to express Fas-ligand following T cell receptor crosslinking. Similar to T cells from normal donors, we found that T cells from synovial fluid or synovium can be induced to express Fas-ligand through CD3 crosslinking or co-culture with phorbol esters and a calcium ionophore *in vitro*. The lack of appropriate Fas-ligand expression suggests that the RA synovium may be an "immune under-privileged" site, where mononuclear cells of affected joints can express high levels of immunostimulatory accessory molecules, including CD95, that commonly are induced upon cellular-immune activation. Without sufficient expression of Fas-ligand, however, lymphocyte activation may perpetuate itself through recurrent cycles of antigen-independent, co-stimulatory, cognate cell-cell interactions. This study reveals that human autoimmune disease may be associated with suboptimal or defective expression of Fas-ligand, suggesting that the regulated expression of this accessory molecule is as critical for immune-system homeostasis in humans as it is in inbred strains of mice.

ALLOGENEIC PLATELETS REQUIRE UNIQUE ANTIGEN PROCESSING MECHANISMS WITHIN RECIPIENT ANTIGEN PRESENTING CELLS (APC) IN ORDER TO STIMULATE ALLOANTIBODY PRODUCTION. Annie Bang, Kevin J. Hicks, Edwin R. Speck, Victor Blanchette, John Freedman and John W. Semple. Division of Hematology, St. Michael's Hospital and The Hospital for Sick Children and The University of Toronto, Toronto, Ontario, Canada.

We have recently shown that the immunogenicity of allogeneic platelets is dependent on stimulation of inducible nitric oxide synthase (iNOS) within recipient antigen presenting cells (APC, Blood 88(8), 1996, in press). To examine platelet processing pathways in recipient APC, splenic adherent macrophages (APC) from BALB/c (H-2^d) mice were incubated (pulsed) with allogeneic C57BL/6 (H-2^b) platelets (10:1; platelet:APC) for 18 hr at 37°C, washed 2x and 10⁶ pulsed-APC were transfused weekly into BALB/c mice. IFN-γ and various drug inhibitors (known to affect antigen processing) were incubated in the platelet/APC cultures. Results show that recipient APC pulsed with syngeneic platelets did not induce formation of anti-donor alloantibodies whereas allogeneic platelet-pulsed APC could stimulate alloantibodies by the 2nd transfusion. Flow cytometric analysis confirmed that the IgG antibodies were specific for cells of the donor MHC (H-2^b) haplotype. When APC were pulsed with allo-platelets in the presence of cytokine or drug, the percentage of mice (N=10) with detectible anti-donor alloantibodies was as follows:

| In vitro Cytokine/Drug | WEEKS OF TRANSFUSIONS | | | |
|----------------------------|-----------------------|-----|-----|-----|
| | Pre | 2 | 4 | 6 |
| None | 0 | 40 | 80 | 100 |
| IFN-γ | 0 | 80 | 100 | 100 |
| Aminoguanidine (AMG, 1 mM) | 0 | 20 | 30 | 40* |
| Colchicine (1 μg/ml) | 0 | 20 | 30 | 30* |
| NH Cl (50 mM) | 0 | 40 | 80 | 100 |
| Chloroquine (100 μM) | 0 | 100 | 100 | 100 |

* mice had low titred antibodies.

These data suggest that recipient APC require iNOS activation (AMG sensitivity) and a colchicine sensitive (phagocytosis), pH-independent (chloroquine/NH Cl-insensitive) cellular compartment in order to optimally process/present platelet antigens and induce anti-donor antibody production.

ADVANTAGES OF HUMAN CD40 ACTIVATED B CELLS OVER DENDRITIC CELLS FOR PRESENTATION OF HUMAN TUMOR ANTIGENS J.L. Schultze¹, S. Michalak², M.J. Seamon³, J.C. Delgado⁴, J.G. Gribben⁵, L.M. Nadler. Dana-Farber Cancer Institute, Boston, MA 02115.

Tumor cells themselves are either inefficient or ineffective antigen presenting cells (APC). One approach to circumvent this obstacle is to use professional APC to present tumor associated peptide antigens (Ag) to attempt to induce T cell mediated anti-tumor specific immunity. Candidate APC include dendritic cells (DC), activated B cells, and monocytes. Based upon their efficiency of Ag presentation, DC have largely been selected as candidates for clinical studies. Since DC's comprise <1% of peripheral blood mononuclear cells (PBMC), leukopheresis or stem cell mobilization are used to obtain sufficient sources of these cells. Although DC's can be expanded in cocktails of cytokines, long term culture (>21d) results in diminished capacity of these cells to function as APC. Considering the difficulty in preparation, expansion, function, and potential cost of using DC as cells to present tumor Ags, we sought alternative sources of professional APC. Human B cells activated by CD40 crosslinking (CD40-B) are highly efficient APCs to present tumor peptide Ag. Although DCs are approximately 5-10 fold more efficient at presenting alloAg, the ease in obtaining large numbers of CD40-B from small quantities of PBMC obviates this problem. CD40-B can also be generated from patients heavily pre-treated with chemotherapy, whereas it is much more difficult to obtain DCs from these patients. From PBMC preparations (n=13), between 83 and 773 (mean 375) fold increase in activated B cells can be generated. Unlike DC, CD40-B can be cultured for more than 65 days (expansion 98,000 fold) without loss in their capacity to present alloAg efficiently to T cells. More importantly, CD40-B cells reproducibly induced a higher peak T cell proliferative response than PBMC DCs cultured in GM-CSF and IL-4 from the same donor. To evaluate their capacity to present MHC class I restricted tumor peptide Ag, CD40-B cells from HLA-A201* healthy donors were pulsed with the melanoma associated tumor Ag tyrosinase peptide YMGMTMNSQV, known to bind to HLA-A201*. When CD8+ T cells (> 99%) were repetitively stimulated with peptide pulsed autologous CD40-B cells, they could kill target cells pulsed with the immunogenic peptide specifically, but not cells alone, allogeneic control targets, autologous B cells pulsed with irrelevant immunogenic peptide or PHA blasts. Finally, unlike DCs, CD40-B cells remain fully functional even in the presence of immunosuppressive cytokines including IL-10 and TGFβ. Considering their ease in isolation, equivalence in Ag presentation, retention of Ag presentation function in long term culture, and resistance to inhibitory cytokines, these studies provide evidence that CD40-B cells should be considered as a potentially superior source of APC to present tumor Ag.

IMMUNE NON-RESPONSIVENESS TO ALLOGENEIC PLATELETS IN MURINE STRAINS WHICH LACK MHC CLASS II I-E MOLECULES IS DUE TO THE PRESENCE OF CD8+ T CELLS. John W. Semple, Edwin R. Speck, Victor Blanchette and John Freedman. Division of Hematology, St. Michael's Hospital and The Hospital for Sick Children and The University of Toronto, Toronto, Ontario, Canada.

BALB/c (H-2^d) and CBA (H-2^k) mice express the full complement of murine MHC class II; both I-A and I-E MHC molecules. When these mice are transfused with 2 x 10⁶ leukoreduced allogeneic platelets, they generate IgG, and IgG anti-donor antibodies by the fifth transfusion. C57BL/6 (BL/6, H-2^b) and ASW (H-2^s) mice on the other hand only express I-A MHC class II molecules; these mice are alloantibody non-responders to all allogeneic platelets (>25 transfusions) tested to date. Spleen cells from the transfused ASW or BL/6 mice could not mediate nitric oxide-dependent cytotoxicity which we have recently demonstrated to be essential for platelet-induced IgG antidonor antibody production (Blood 88(8), 1996 in press) nor did they show any donor-specific CTL-mediated cytotoxicity. Because T cells may be involved with the platelet immune non-responsiveness, we used BL/6 CD4^{-/-} and CD8^{-/-} transgenic knockout mice as platelet recipients. CD4^{-/-} mice could not respond humorally to any allogeneic platelet donor tested during a 10 week transfusion protocol whereas the CD8^{-/-} mice responded by the 3rd platelet transfusion. The CD8^{-/-} alloantibodies were predominantly IgG_{2b} (without IgG₁), donor MHC specific, and of relatively high titre (range: 1:200-3200). These data suggest that IgG immune responsiveness against allogeneic platelets is differentially regulated by MHC class II molecules, i.e. I-E with I-A expression stimulates immunity whereas I-A expression alone suppresses antibody production via a CD8+ T cell pathway. In addition, the data suggests I-A restriction of platelet alloantigens may be responsible for a preferential IgG_{2a} (Th1) immune response.

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Dendritic Cell Secretion of IL-15 Is Induced by Recombinant huCD40LT and Augments the Stimulation of Antigen-Specific Cytolytic T Cells¹

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Dendritic cells (DC) are professional antigen-presenting cells which stimulate strong proliferative and cytolytic T cell responses. Stimulation of CD40 on dendritic cells by its ligands and anti-CD40 antibodies induces maturation and enhances DC stimulatory ability. In order to understand the mechanism by which ligand:CD40 interactions augment DC function, we assessed the role of T cell stimulatory cytokines IL-12 and IL-15 in the function of DC stimulated with soluble trimeric CD40L, a recombinant fusion protein incorporating three covalently linked extracellular CD40L domains (huCD40LT). Peripheral blood derived DC treated with huCD40LT and/or IFN- γ were used to stimulate T cell responses *in vitro* to specific antigens. DC treated with huCD40LT or IFN- γ /huCD40LT stimulated enhanced T cell proliferation to CASTA, a soluble protein from *C. albicans*, induced T cells with augmented antigen-specific lysis, and increased the yield of antigen-specific IFN- γ -producing T cells. IL-15 production by DC was enhanced in cultures treated with huCD40LT and correlated with expansion of antigen-specific cytolytic T cells. Addition of a neutralizing anti-IL-15 monoclonal antibody inhibited the expansion of viral and tumor antigen-specific T cells stimulated by IFN- γ and huCD40LT-treated DC. In contrast, this enhanced stimulatory ability of DC did not appear to depend on synthesis of IL-12 since huCD40LT treatment stimulated the generation of antigen-specific cytokine producing and cytolytic T cells without increased IL-12 production. Addition of anti-IL-12 monoclonal antibody did not inhibit expansion of these cells. These data suggest that production of IL-15 but not IL-12 is an important factor in the enhanced immunostimulatory ability of huCD40LT-treated DC. © 1999 Academic Press

Key Words: dendritic cells; IL-15; CD40L; tumor antigens; antigen presentation.

INTRODUCTION

The dendritic cell is a professional antigen presenting cell (APC)² which induces the formation of antigen-specific immune responses in naive T cells (1, 2). DC-mediated immune responses include the development of primary and secondary T cell helper and cytolytic immune responses, T-cell-dependent antibody production, and induction of tolerance (1–4). Vaccination with peptide-pulsed DC has been shown to induce anti-viral and anti-tumor T cell responses in mice and causes regression of established tumors (5, 6). DC capture and process antigen, become activated in tertiary lymphoid tissue, migrate to secondary lymphoid tissues, and stimulate T-cell-dependent immune responses (1, 7, 8). DC exposed to LPS and proinflammatory cytokines mature, demonstrate decreased capacity for new antigen presentation, increase their expression of immunomodulatory cell surface markers, and have enhanced ability to stimulate immune responses (9–13). Tumor cell-induced defects in DC maturation and function have been reported (14) and may be due to tumor cell production of vascular endothelial growth factor (15). DC have been shown to produce a variety of cytokines during maturation which may be important for their immune activating function, including IL-12 and IL-15 (16–19).

² Abbreviations used: DC, dendritic cell; APC, antigen presenting cell; PBMC, peripheral blood mononuclear cells; IFN- γ , interferon-gamma; LPS, lipopolysaccharide; NK, natural killer; CTL, cytolytic T cell; MLR, mixed lymphocyte reaction; TNF, tumor necrosis factor; IL-, interleukin; huCD40LT, soluble trimeric CD40 ligand fusion protein; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; GM-CSF, granulocyte-macrophage colony stimulation factor; CASTA, Candida skin test antigen; HLA, human leukocyte antigen; IL-15, interleukin.

¹ This work was supported in part by National Cancer Institute Grant 5P30-CA 14089 and by Food and Drug Administration Grant FD-R-001101-1 to J.S.W. J.S.K. was supported by a grant from RPR-Gencell, Inc., and by NIH Traineeship 2T32AI07078-16A2.

IL-12, a heterodimeric 70-kDa cytokine, is an important mediator in the establishment of both antigen-specific T cell and nonspecific NK cell immune responses (16, 20, 21). Dendritic cell (17, 18) production of IL-12 in response to bacterial products and inflammatory mediators may be important for the stimulation of IFN- γ -producing cells in antigen-specific cytolytic T cell responses (18, 22–24) and has been hypothesized to play a role in mature DC stimulation of allogeneic proliferative and cytolytic immune responses (25).

IL-15 is a recently identified cytokine which is functionally similar to IL-2 and stimulates both T and NK cells (26–28). IL-15 has been demonstrated to increase HIV-specific CTL *in vitro* (29) and is associated with increased T cell response to the intracellular pathogen *M. leprae* (30). IL-15 can act as a chemoattractant for T cells and induces effector mechanisms in both cytolytic T cells and NK cells (26, 28, 31). IL-15 is produced by dendritic cells after exposure to bacterial products and induction of phagocytosis (19, 32) but not by T cells.

The interaction between cell surface protein CD40 on antigen presenting cells and its ligand gp39 is critical to the development of T-cell-dependent humoral immune responses (33, 34). The importance of this interaction in the generation of cell-mediated and humoral immunity is well documented (35, 36), and recent data suggest that CD40/CD40L interactions play an important role in DC-induced T cell activation (24, 37). In this work we show that treatment of DC with recombinant huCD40LT and other immunostimulatory molecules promotes the expansion of antigen-specific CTL from normal donors and patients with melanoma. The mechanism by which peptide-pulsed DC treated with huCD40LT stimulated antigen-specific reactivity has been explored by analysis of DC cytokine production and its correlation with the immunostimulatory ability of mature DC.

RESULTS

Increased proliferation by melanoma patient PBMC in response to CASTA pulsed dendritic cells treated with huCD40LT. Dendritic cells derived from CD34⁺ progenitors (38) or peripheral blood mononuclear cell precursors (9, 39) have been grown from normal human donors in the presence of human or fetal calf serum with TNF- α and GM-CSF or IL-4 and GM-CSF, respectively. In our studies, DC from normal donors and melanoma patients were expanded in AIM-V serum-free medium supplemented with IL-4 and GM-CSF (DC) resulting in cultures which were 75 to 95% HLA-DR and CD86 positive and CD14 and CD19 negative. DC from normal donors and melanoma patients had characteristic dendritic morphology and demonstrated no significant differences in expression patterns of the aforementioned surface proteins (data not shown).

Membrane-bound CD40L and other immunostimulatory molecules have been shown to augment alloreactive proliferation stimulated by DC (24, 37), therefore we expected that DC treatment with recombinant huCD40LT would enhance the proliferation of autologous PBMC in response to soluble foreign antigens. We assayed the ability of DC treated with huCD40LT +/- IFN- γ to stimulate proliferation in response to CASTA, a *Candida albicans* protein extract known to stimulate good DTH responses (40) (Fig. 1). DC treated with huCD40LT stimulated a significant increase in proliferation at 72 h in response to CASTA in all normal donors and patients when compared to DC grown in GM-CSF and IL-4 (control DC). In all six melanoma patients as well as two of the three normal donors (ND2 and ND3), addition of IFN- γ enhanced proliferation beyond that stimulated by huCD40LT-treated DC. Exposure to DC to IFN- γ alone did not significantly alter proliferation in normal donors or melanoma patients. These findings suggest that huCD40LT alone or combined with IFN- γ augments the capacity of DC from normal donor and melanoma patient PBMC to stimulate proliferative T cell responses to a soluble protein.

Development of Flu antigen-specific CTL is augmented by dendritic cells treated with huCD40LT. Since exposure of DC to CD40L-expressing cells also enhanced the ability of DC to stimulate allogeneic cytotoxicity (25) we hypothesized that synthetic recombinant immunomodulatory proteins like huCD40LT would increase the capability to stimulate MHC Class I restricted CTL responses. To test this hypothesis 9-day DC were pulsed with the HLA-A2 restricted Flu-M1 peptide (NP 66-75), then treated with IFN- γ and/or huCD40LT, and used to stimulate autologous PBMC from HLA-A2⁺ normal donors (Figs. 2 and 3). Ten days after primary *in vitro* stimulation with DC treated with huCD40LT +/- IFN- γ autologous PBMC effector cells demonstrated antigen-specific cytotoxicity (42 and 47%, respectively, at E:T ratio of 30:1) while effector cells stimulated with DC exposed to IFN- γ or grown in GM-CSF and IL-4 (control DC) induced less than 25% specific lysis (Fig. 2A). Background lysis of unpulsed targets was less than 10% at 30:1 E:T ratios. After a second *in vitro* stimulation, peptide-pulsed DC from all treatment groups stimulated comparable levels of antigen-specific lysis (33–39%) at 30:1 E:T ratio (Fig. 2B). Identical effector cells stimulated with control DC or DC treated with huCD40LT, IFN- γ , or the combination of huCD40LT and IFN- γ were used in an IFN- γ ELISPOT assay to determine whether the enhanced cytotoxicity correlated with the number of Flu-M1-specific IFN- γ -producing T cells (Fig. 3). Treatment of DC with huCD40LT (1850 +/- 125) or IFN- γ plus huCD40LT (2310 +/- 629) resulted in significant increases of Flu-M1-specific IFN- γ -producing effectors

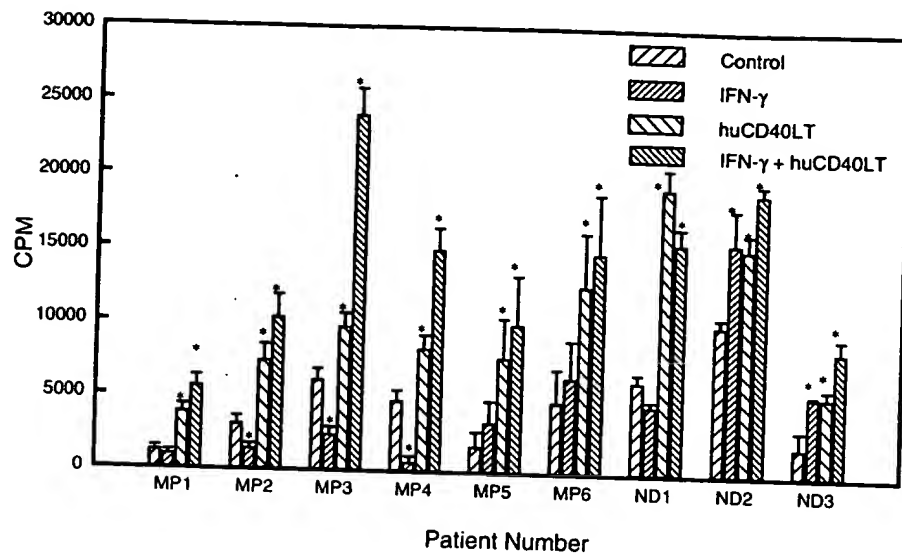


FIG. 1. huCD40LT enhances DC-induced proliferation to CASTA. DC from six melanoma patients (MP1-MP6) and three normal donors (ND1-ND3) were treated with IFN- γ , huCD40LT, or IFN- γ /huCD40LT and pulsed overnight with 10 μ g/ml CASTA. DC were harvested and used as stimulators of autologous PBMC in a 72-h proliferation assay. Proliferation was measured by tritiated thymidine incorporation. Conditions resulting in changes in proliferation which are significantly different from control for each patient are denoted with *. Statistical significance was established using Dunnett's method of multiple comparisons at the level of $P < 0.05$ (46) and a one-way analysis of variance with 95% confidence intervals.

compared with control DC (33 \pm 106). Exposure to IFN- γ alone (480 \pm 50) yielded a modest increase in stimulation compared with control DC. Addition of anti-CD40 antibody M2 to huCD40LT and IFN- γ -treated DC blocked the enhanced effector cell production (data not shown). These results indicate that huCD40LT treatment resulted in DC from normal donors with enhanced ability to stimulate antigen-spe-

cific cytolytic and IFN- γ -secreting T cells after a single *in vitro* stimulation compared with control or IFN- γ -treated DC.

huCD40LT treatment of dendritic cells stimulates tumor antigen-specific CTL from melanoma patients. To test whether treatment of patient DC with IFN- γ and huCD40LT resulted in stimulation of increased

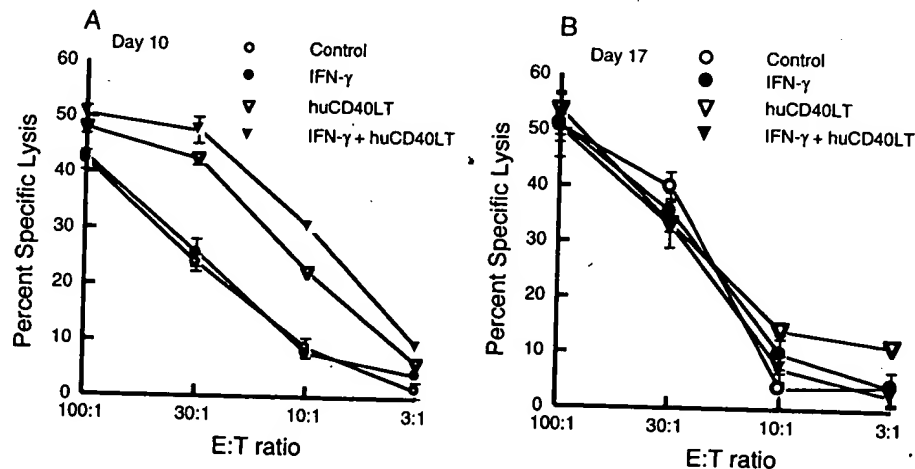


FIG. 2. huCD40LT stimulates DC-induced viral antigen-specific lysis. DC were treated with IFN- γ or huCD40LT and pulsed overnight with Flu-M1 peptide as described under Materials and Methods. DC were harvested and used as stimulators of autologous PBMC for antigen-specific CTL growth. Flu-M1 antigen-specific effector cells were grown for 10 days following primary *in vitro* stimulation (A) or 17 days after receiving a second *in vitro* stimulation with untreated DC on day 10 (B). Cultures were harvested and the isolated effector cells were assayed for their ability to induce Flu-M1 specific lysis. Specific lysis was determined by percentage lysis of 51 Cr-labeled Flu-M1 peptide-pulsed T2 cells minus percentage lysis of gp100 peptide-pulsed T2 cells, which did not exceed 10% at 30:1 E:T ratio. Representative data are presented from experiments which have been repeated in two normal donors.

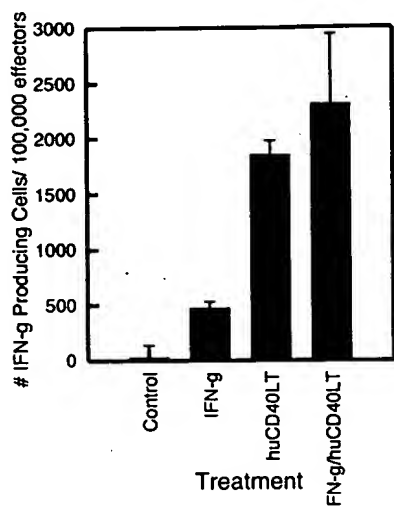


FIG. 3. huCD40LT enhances DC-induced expansion of viral antigen-specific IFN- γ -producing cells. DC were treated with IFN- γ or huCD40LT and pulsed overnight with Flu-M1 peptide as described under Materials and Methods. DC were harvested and used as stimulators of autologous PBMC for antigen-specific CTL growth. Flu-M1 antigen-specific effector cells were grown for 10 days following primary *in vitro* stimulation. Cultures were harvested and the isolated effector cells were assayed for number of IFN- γ -producing cells per 100,000 effectors as described under Materials and Methods. Values represent the number of IFN- γ -positive cells responding to Flu-M1 peptide-pulsed T2 cells minus percentage lysis of gp100 peptide-pulsed T2 cells. Representative data are presented from experiments which have been repeated in four normal donors and one melanoma patient.

tumor antigen-specific CTL activity, MART-1 peptide-pulsed DC treated with IFN- γ , huCD40LT, or the combination were used to stimulate autologous melanoma patient PBMC. As with Flu-M1, MART-1 peptide-pulsed DC treated with huCD40LT alone or IFN- γ and huCD40LT stimulated antigen-specific cytotoxicity after 9 days (17 and 23%, respectively, at 100:1 E:T ratio) (Fig. 4A). Background lysis of unpulsed targets was less than 5% at all E:T ratios tested. Effectors stimulated with IFN- γ -treated or untreated DC did not show significant MART-1 specific cytotoxicity above background 9 days after stimulation. Identical effector cells stimulated with control, huCD40LT-, IFN- γ , or huCD40LT/IFN- γ -treated patient DC were used in an IFN- γ ELISPOT assay to confirm that enhanced cytotoxicity correlated with the number of MART-1-specific IFN- γ -producing T cells (Fig. 4B). Treatment of DC cultures with huCD40LT (960 \pm 45) or IFN- γ and huCD40LT (906 \pm 45) but not IFN- γ alone (320 \pm 92) resulted in greater than a fourfold increase of MART-1-specific IFN- γ -producing effectors compared with control DC (200 \pm 60). To understand the mechanism of the increased immunostimulatory ability of peptide-antigen-pulsed DC treated with huCD40LT we examined the production of cytokines implicated in the formation of T-cell-mediated immune

responses by DC treated with huCD40LT with or without IFN- γ .

Exposure of DC to huCD40LT alone stimulates increased IL-15 production. In order to determine whether IL-15, a T cell stimulatory molecule secreted by DC (19, 32), contributed to the enhanced stimulation of antigen-specific CTL by huCD40LT-treated DC, supernatants were harvested at 24-h timepoints after day 10 medium change of control, huCD40LT-, and/or IFN- γ -treated DC, concentrated fivefold, and assayed for IL-15 production (Fig. 5A). Peak IL-15 production by DC was in the 0- to 24-h supernatant and increased threefold after treatment with huCD40LT (58 pg/ml) or IFN- γ /huCD40LT (97 pg/ml) but not in response to treatment with IFN- γ (15 pg/ml) alone compared with control DC (10 pg/ml). Addition of anti-CD40 antibody abrogated the increase in IL-15 production by huCD40LT- and IFN- γ -treated DC (data not shown). IL-15 production by DC treated with huCD40LT with or without IFN- γ correlated with the increased antigen-specific immunostimulatory ability of DC and was seen with DC from normal donors and patients.

IFN- γ plus huCD40LT but not huCD40LT alone induces increased synthesis of IL-12 from peripheral blood-derived DC. DC grown from PBMC-derived monocytes of normal individuals have been shown to produce IL-12 in response to treatment with J558L cells expressing membrane-bound CD40L. In those studies DC production of IL-12, a cytokine which has been shown to enhance cell-mediated immune responses (16, 20, 21), correlated with an increased ability to generate alloreactive proliferative and cytolytic responses (24). To determine whether IL-12 is produced by DC grown with and without huCD40LT and/or IFN- γ , DC supernatants from melanoma patients and normal donors were harvested from 4 to 120 h after replacement of AIM V medium on day 10 of culture. Control, IFN- γ , huCD40LT, or IFN- γ /huCD40LT DC were assayed for IL-12 production by ELISA (Fig. 5B). These data indicate that DC cultures derived from different melanoma patient or healthy donor PBMC produce IL-12 only in response to the combination of a trimeric huCD40LT fusion protein and IFN- γ (187 pg/ml) but not to either agent alone (<6 pg/ml) and that IL-12 production rapidly declines without further stimulation during the initial 12 h after washout.

Neutralizing antibodies to IL-12 and IL-15 block DC-induced proliferation to CASTA. To determine the impact of IL-12 and IL-15 production by DC on the enhanced proliferation demonstrated by IFN- γ /huCD40LT-treated CASTA-pulsed DC we measured the effect of IL-12 and IL-15 neutralization on 72-h proliferation to CASTA (Fig. 6). Addition of a neutralizing anti-IL-12 monoclonal antibody resulted in

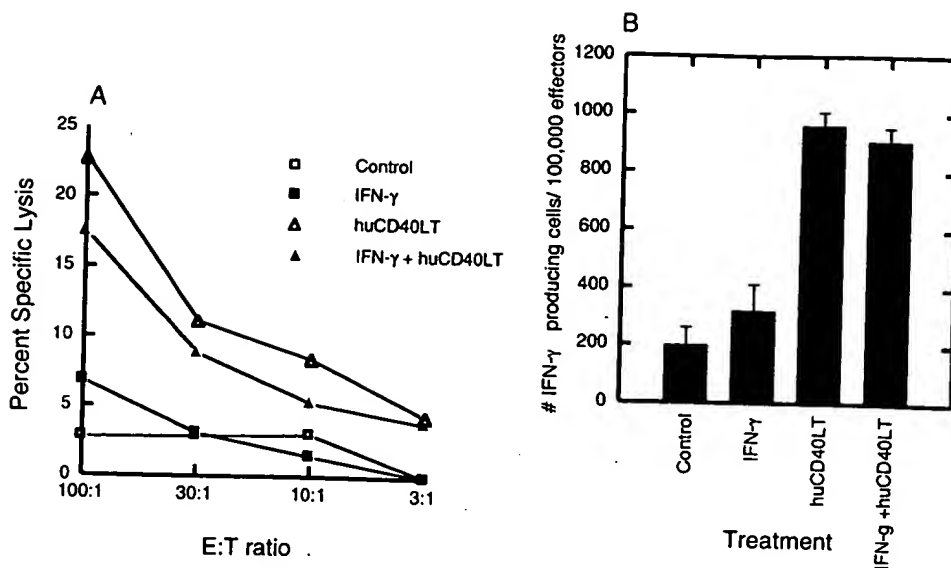


FIG. 4. Soluble CD40L fusion protein alone or in combination with IFN- γ -treated DC increases MART-1 tumor antigen-specific CTL. Melanoma patient DC were treated with IFN- γ , huCD40LT, or IFN- γ and huCD40LT and pulsed overnight with MART-1 peptide. DC were harvested and used as stimulators of autologous PBMC for antigen-specific CTL growth. MART-1 antigen-specific effector cells were grown for 9 days following primary *in vitro* stimulation with DC. (A) Tumor antigen-specific lysis was determined by chromium release from ^{51}Cr -labeled MART-1 peptide-pulsed T2 target cells minus percentage lysis of gp100 peptide-pulsed T2 cells, which did not exceed 5% at any E:T ratio as described under Materials and Methods. (B) Number of effector cells which specifically responded to the MART-1 tumor antigen with production of IFN- γ was assayed by ELISPOT assay as described under Materials and Methods. Values represent the number of IFN- γ -positive cells responding to MART-1 peptide-pulsed T2 cells minus percentage lysis of gp100 peptide-pulsed T2 cells. Representative data are presented from an experiment which has been repeated in four patients.

reduction of the average tritiated thymidine incorporation in two normal donors by $40 \pm 9\%$, anti-IL-15 antibody reduced proliferation minimally by $20 \pm 10\%$, and both anti-IL-12 and anti-IL-15 reduced proliferation by $55 \pm 2.5\%$ while an isotype control antibody did not alter proliferation. These data suggest that IL-12 but not IL-15 plays a role in DC-mediated proliferation to CASTA, a soluble *C. albicans* protein.

An anti-IL-15 but not an anti-IL-12 monoclonal antibody blocks the expansion of HLA-A2-restricted antigen-specific T cells stimulated by IFN- γ /huCD40LT-treated DC. To examine whether production of IL-15 and/or IL-12 by DC is responsible for the enhancement of tumor antigen-specific CTL activity we blocked IFN- γ /huCD40LT-treated DC-stimulated CTL cultures using anti-IL-15 and anti-IL-12 monoclonal antibodies and measured the number of IFN- γ -producing T cells in an ELISPOT assay. Blocking antibodies as well as an isotype control antibody were added to appropriate wells at the establishment of the CTL cultures. Ten days later MART-1- (Fig. 7A) or Flu-M1- (Fig. 7B) specific T cells were measured. Treatment of MART-1 peptide-pulsed DC with IFN- γ /huCD40LT resulted in an increase in the number of specific IFN- γ -producing cells from 90 ± 55 (control DC) to 650 ± 86 . Addition of anti-IL-15 blocking antibody decreased

the number of IFN- γ -producing cells to 120 ± 75 while addition of anti-IL-12 antibody and an isotype control antibody did not significantly block expansion of antigen-specific IFN- γ -producing cells (493 ± 63 and 530 ± 11 , respectively). Similarly, treatment of Flu-M1 peptide-pulsed DC with IFN- γ /huCD40LT resulted in an increase in the number of specific IFN- γ -producing cells from 33 ± 106 (control DC) to 2310 ± 629 . Addition of anti-IL-15 blocking antibody decreased the number of IFN- γ -producing cells to 825 ± 214 while addition of anti-IL-12 antibody and an isotype control antibody did not significantly block expansion of Flu-M1 antigen-specific IFN- γ -producing cells (1947 ± 268 and 2607 ± 548 , respectively). Addition of neutralizing anti-IL-15 antibody to control DC did not decrease effector cell stimulation (data not shown).

In order to determine if IL-15 produced by DC resulted in the expansion of antigen-specific T cells with cytolytic activity, effectors were also tested for their ability to lyse appropriate target cells in a chromium release assay, shown in Fig. 8. Treatment of the Flu-M1 peptide-pulsed DC with IFN- γ /huCD40LT resulted in an increase in the Flu-specific lysis from 23% (control DC) to 47% at a 30:1 E:T ratio. Addition of anti-IL-15 blocking antibody decreased Flu-M1 specific lysis to 12% at a 30:1 E:T

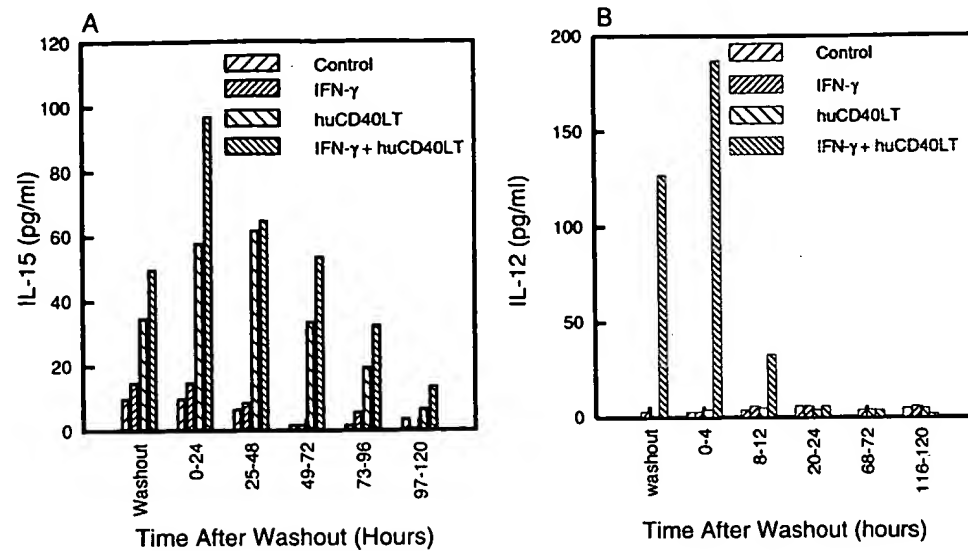


FIG. 5. IL-12 and IL-15 production by DC is enhanced by immunomodulatory agents. DC were grown for 9 days and then exposed to IFN- γ , TNF- α , huCD40LT, and IFN- γ /huCD40LT for 18 h. Supernatants were collected as described and assayed by sandwich ELISA for the presence of IL-15 (A) or IL-12 (B). IL-15 release in A is from fivefold concentrated supernatants collected every 24 h after the day-10 medium change. IL-12 release in B are supernatants collected from 4 to 120 h after day-10 medium change. Representative data are presented from an experiment which has been repeated in four patients and two normal donors.

ratio while addition of anti-IL-12 neutralizing antibody and an isotype control antibody did not significantly block Flu-M1 specific lysis (49 and 43%, respectively, at a 30:1 E:T ratio). Background lysis of unpulsed targets was less than 10% at 30:1 E:T ratios. These data suggest that increased production of IL-15 contributes to huCD40LT/IFN- γ -treated DC stimulation of tumor and viral antigen-specific T cells from normal donors and cancer patients.

DISCUSSION

Cella *et al.* established that monocyte-derived DC cocultured with J558L cells expressing CD40L stimulated increased levels of proliferation in an allogeneic MLR (24). In this study, we have employed a construct which covalently links three CD40L extracellular domains in one recombinant molecule to stimulate human dendritic cells. The role of DC in the generation of antigen-specific immune responses has been the focus of extensive study, and published data suggest that DC have the ability to prime effective viral and tumor antigen-specific immunity (6, 41). APC capable of stimulating tumor antigen-specific immune responses *in vitro* might facilitate the generation of anti-tumor immune responses *in vivo* after adoptive transfer (6, 41). The melanoma antigen MART-1 (aa27-35) (42, 43) and the viral-specific antigen Flu-M1 (aa66-75) (44), encoding 9 amino acid HLA-A2-restricted epitopes, were chosen as model antigens for assessment of MHC Class I restricted immune responses, and

CASTA, a *C. albicans* protein which generates potent DTH responses were used for assessment of proliferative responses to soluble protein. MART-1 responses were tested in melanoma patients and Flu-M1 responses in normal donors and patients. Our studies demonstrate that recombinant huCD40LT induces enhanced DC-stimulated proliferation in response to CASTA in normal donors and melanoma patients. The addition of IFN- γ to huCD40LT-treated DC resulted in increased mean proliferation to CASTA.

Our studies also demonstrate that addition of recombinant huCD40LT enhanced the ability of peptide-pulsed DC to stimulate the expansion of antigen-specific cytotoxic and IFN- γ -producing T lymphocytes from autologous PBMC *in vitro*. This immunostimulatory effect of huCD40LT permitted the detection of antigen-specific CTL within 10 days of primary *in vitro* stimulation of the PBMC as had been demonstrated for allogeneic CTL (25). In contrast to the effect of huCD40LT upon CASTA-induced proliferation, treatment of peptide-pulsed DC with huCD40LT stimulated similar levels of antigen-specific cytotoxicity with or without IFN- γ . These data show that treatment of DC with huCD40LT resulted in the production of APC with increased capacity for stimulation of viral and tumor antigen-specific immune responses.

Production of cytokines by APC is an important mechanism for the generation and orientation of immune responses. IL-15 and IL-12 are produced by activated macrophages and dendritic cells and their

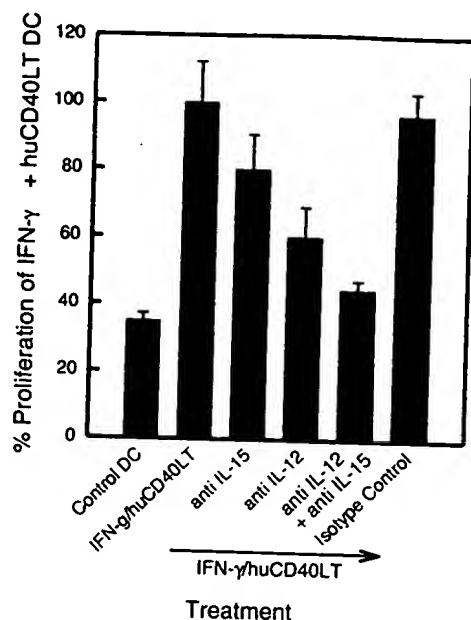


FIG. 6. Neutralization of IL-12 and IL-15 decreases proliferation of autologous PBMC stimulated by CASTA-pulsed DC. DC from normal donors were treated with IFN- γ and huCD40LT and pulsed overnight with CASTA and used to stimulate proliferation by autologous PBMC. Proliferation assays were treated with anti-IL-15, anti-IL-12, or a control IgG₁ monoclonal antibody. Proliferation was measured by tritiated thymidine incorporation and is reported as percentage of IFN- γ + huCD40LT-treated DC-stimulated control. Reported values represent the average \pm 95% confidence intervals of a representative experiment performed in two normal donors.

synthesis is enhanced in response to bacterial products and by induced phagocytosis (17, 19, 21, 24, 28). Production of IL-15 by antigen presenting cells is important for the generation of cell-mediated immune responses (29, 30) and IL-15 production by DC has been demonstrated to enhance chemotaxis of T cells (16–19). IL-15 binds to a receptor composed of the β and γ chains of the IL-2 receptor to exert its function which overlaps the actions of IL-2. IL-15 is induced by DC after phagocytosis or in response to LPS, suggesting that it plays an early role in the induction and amplification of cell responses. Its chemotactic effects when produced by DC would be consistent with a role in early T cell activation. In our experiments ligation of CD40 on DC by recombinant huCD40LT with or without IFN- γ resulted in a threefold increase in IL-15 production. In order to test the hypothesis that IL-15 plays a role in DC stimulation of MHC Class I restricted T cell responses, we demonstrated that IL-15 but not IL-12 neutralization in DC-stimulated T cell cultures abrogated the expansion of antigen-specific IFN- γ -producing and cytolytic T cells from normal donors and melanoma patients. We concluded that there is a direct link between IL-15 production by DC and the stimulation of MHC Class I restricted antigen-

specific T cells and that production of IL-15 can be stimulated by ligation of CD40 on DC *in vitro* by a recombinant CD40L trimer fusion protein. IL-15 may enhance DC-mediated stimulation of effector T cells through direct activation of effector mechanisms in an IL-2-like role or alternatively enhance chemotaxis of T cells.

Monocyte-derived DC (MODC) isolated from the peripheral blood of normal donors have been shown to produce IL-12 in response to coculture with cells expressing membrane-bound CD40L on J558 transfectants (24, 25). In our study DC did not produce IL-12 in response to ligation by recombinant huCD40LT but required additional stimulation by IFN- γ . This difference from published data could be attributed to at least four different factors: (1) the CD40L-expressing cells in the literature are producing IFN- γ , (2) interactions other than CD40/CD40L between the DC and the J558 cell may modulate the activation of DC, (3) recombinant huCD40LT fusion protein may not induce the same effects as aggregated membrane-bound CD40L, and (4) the different media and culture conditions used in our studies resulted in DC in a different activation state than the MODC produced by Cella *et al.* Our data demonstrate that while IL-12 production by DC correlated with increased proliferation in response to CASTA, it did not coincide with production of antigen-specific cytotoxicity or IFN- γ -secreting MHC Class I restricted antigen-specific T cells. Furthermore, IL-12 neutralization decreased autologous proliferation in response to CASTA but had no effect on MART-1 or Flu-M1 antigen-specific effector T cell expansion. However, exogenous IL-12 has been shown to increase the production of cytolytic immune responses stimulated by DC (data not shown) and to the stimulation of CASTA-dependent proliferation. IL-12 produced by DC may be responsible for the stimulation of NK or CD4⁺ T cells which may aid in the stimulation of antigen-specific CTL. Maturation of DC with huCD40LT may bypass the requirement for CD4⁺ and NK cell help in the expansion of class I restricted CTL. This could explain the delay observed in CTL activation by untreated DC until after a second restimulation. Studies investigating the role of IL-15, IL-12, and other cytokines in the stimulation of cell-mediated responses augmented by MHC Class II help are being performed in our laboratory.

In this study, we have also demonstrated that dendritic cells from normal donors or patients with melanoma can stimulate potent anti-viral or anti-tumor T cell responses *in vitro* which are amplified by recombinant huCD40LT and depend on production of IL-15. This result suggests that viral or tumor antigen peptide-pulsed dendritic cells grown from normals or melanoma patient PBMC treated with huCD40LT and

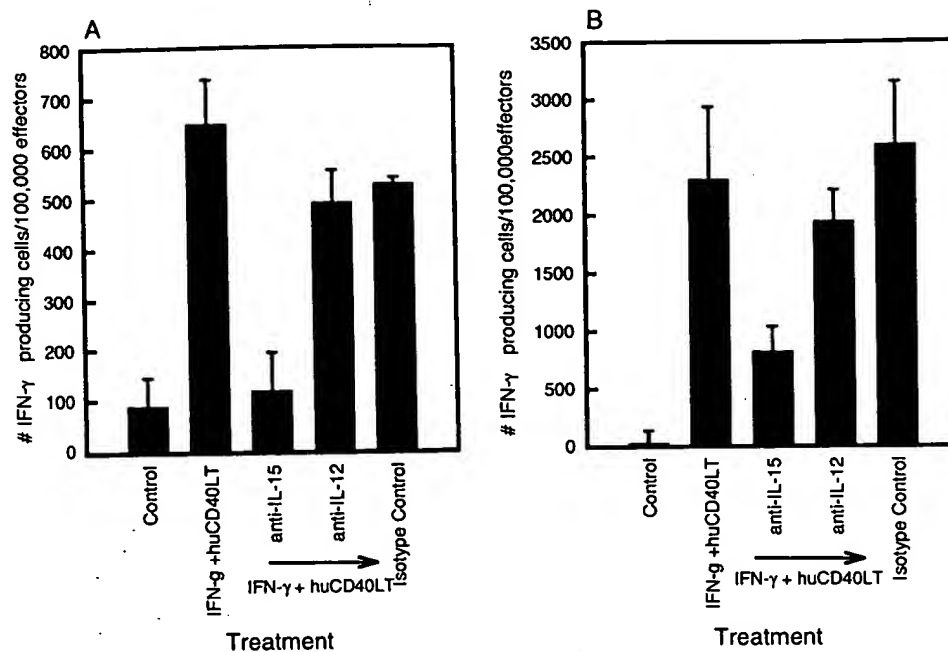


FIG. 7. An anti-IL-15 but not an anti-IL-12 neutralizing monoclonal antibody inhibits the expansion of antigen-specific IFN- γ -producing cells by IFN- γ /huCD40LT-treated DC. DC were treated with IFN- γ and huCD40LT and pulsed overnight with MART-1 (A) or Flu-M1 (B) peptide. DC were harvested and used as stimulators of autologous PBMC for antigen-specific CTL growth. IFN- γ /huCD40LT-treated DC-stimulated CTL cultures were treated with anti-IL-15, anti-IL-12, or a control IgG, monoclonal antibody. Antigen-specific effector cells were grown for 9 days following primary *in vitro* stimulation with DC. The number of antigen-specific IFN- γ -producing cells per 100,000 effector cells was assayed by ELISPOT assay as described under Materials and Methods. Values represent the number of IFN- γ -positive cells responding to MART-1 or Flu-M1 peptide-pulsed T2 cells minus those responding to gp100 peptide-pulsed T2 cells. Representative data are presented from an experiment which has been repeated for MART-1 in three patients and Flu-M1 in four normal donors and one melanoma patient.

expressing IL-15 may be effective as a vaccine to augment anti-viral or anti-tumor immunity. This preclinical idea will be tested in an upcoming clinical trial at our institution in patients with melanoma.

MATERIALS AND METHODS

Collection and purification of mononuclear cells. Peripheral blood mononuclear cells were isolated from leucopheresis specimens of HLA-A2⁺ normal donors or melanoma patients who had their tumor resected and were rendered free of detectable disease. PBMC were enriched by Ficoll-Hypaque density gradient purification and aliquoted and frozen at 5×10^7 cells/ml in 50% AIM V medium (Gibco, Grand Island, NY), 40% heat-inactivated human AB sera (Omega Scientific, Irvine, CA), and 10% DMSO (Sigma, St. Louis, MO). Cell yields were greater than 3×10^9 PBMC per leucopheresis.

Cytokines, cell lines, and reagents. Recombinant IL-4 (6.35×10^7 IU/mg) and GM-CSF (1.35×10^8 IU/mg) used for the production of DC were kindly provided by Dr. Sathwant Narula, Schering-Plough Research Institute (Kenilworth, NJ). Recombinant soluble CD40L trimeric fusion protein was gener-

ously provided by Immunex Corporation (Seattle, WA). Recombinant human IFN- γ was kindly provided by Medarex Corporation (Annandale, NJ). Recombinant human IL-2 was generously provided by Chiron Therapeutics (Emeryville, CA). Recombinant human IL-7 was kindly provided by Sanofi Pharmaceuticals (Labège, France). Antigenic peptides MART-1²⁷⁻³⁵ (AAGIGILTV) (45) and Flu-M1⁵⁸⁻⁶⁶ (GILGFVFTL) (44) were synthesized on a solid state peptide synthesis machine at the USC/Norris Cancer Center microchemical core synthesis facility and reconstituted in 100% DMSO (10 mg/ml). CASTA, a mixture of Candida-associated proteins, was purchased from Greer Laboratories (Lenoir, NC) (40). T2 cells expressing HLA A2.1 were a kind gift of Dr. Franco Marincola, NCI.

Growth of dendritic cells. Dendritic cells were prepared from peripheral blood mononuclear cells by a modification of the method of Romani *et al.* (39). PBMC were thawed and allowed to adhere to plastic for 1 h. Nonadherent cells were removed with agitation. Adherent cells were grown in AIM V medium (Gibco) containing GM-CSF (800 U/ml) and IL-4 (1000 U/ml) (AIM V-DC medium) for 10 days receiving fresh medium and cytokine on day 7. DC cultures were exposed to IFN- γ (250 U/ml), huCD40LT (1.5

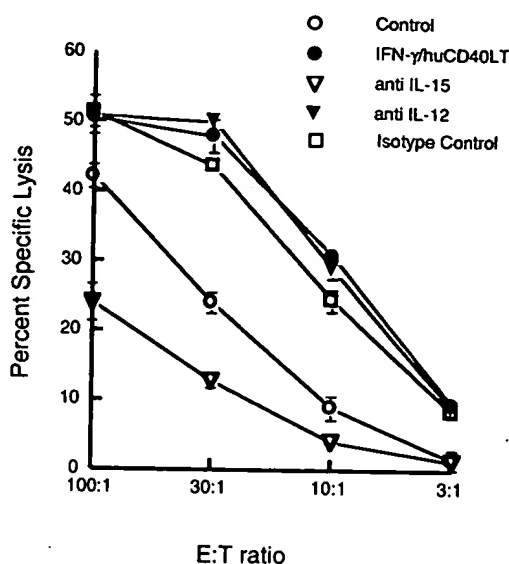


FIG. 8. An anti-IL-15 but not an anti-IL-12 neutralizing monoclonal antibody inhibits the expansion of antigen-specific CTL by IFN- γ /huCD40LT-treated DC. DC were treated with IFN- γ and huCD40LT, pulsed overnight with Flu-M1 peptide, and used to stimulate autologous PBMC for antigen-specific CTL growth. IFN- γ /huCD40LT-treated DC-stimulated CTL cultures were treated with anti-IL-15, anti-IL-12, or a control IgG₁ monoclonal antibody. Tumor antigen-specific lysis was determined by chromium release from ^{51}Cr -labeled Flu-M1 peptide-pulsed T2 cells minus percentage lysis of gp100 peptide-pulsed T2 cells, which did not exceed 10% at 30:1 E:T ratio as described under Materials and Methods. Representative data are presented from an experiment which has been repeated in two normal donors.

$\mu\text{g/ml}$), and/or TNF- α (75 U/ml) for the last 18 h of 10-day cultures.

Proliferation assay. DC were pulsed with 10 $\mu\text{g/ml}$ antigen 20 h prior to harvest and IFN- γ , huCD40LT, or IFN- γ and huCD40LT were added 18 h prior to harvest. DC stimulators (2.5×10^4) were irradiated with 6000 R and incubated with 1.5×10^5 PBMC responders in 96-well plates for 72 h. Tritiated thymidine (1 μCi) (NEN, Boston, MA) was added to each well for the last 18 h of the assay. Cells were harvested onto glass fiber filters using a Model 12010 cell harvester (Skatron, Sterling, VA) and counted in a Model C1600 liquid scintillation counter (Packard Instruments, Palo Alto, CA). Statistical significance was established using Dunnett's method of multiple comparisons at the level of $P < 0.05$ (46) and a one-way analysis of variance of the logarithm transformation of all cpm measurements. Proliferation is reported as mean cpm of five replicate wells above background with 95% confidence intervals. Anti-IL-12 (15 $\mu\text{g/ml}$) and anti-IL-15 (10 $\mu\text{g/ml}$) neutralizing monoclonal antibodies or an isotype control antibody were added at the induction of each proliferation assay for blocking studies.

Cytokine ELISA. IL-12 (p70) and IL-15 production by DC cultures was measured by sandwich ELISA according to the manufacturer's specifications (R&D Systems, Minneapolis, MN). Dendritic cells were cultured in AIM V-DC medium for 10 days to a density of 10^5 cells in 2 ml of medium per well of a 6-well plate. On day 9 IFN- γ and huCD40LT were added to specified cultures. On day 10 culture supernatants were removed and replaced with 2 ml of fresh AIM-V medium. Supernatant samples were harvested at the indicated time points, then aliquoted and frozen at -80°C until analysis. IL-15 supernatants were fivefold concentrated prior to analysis using microcon 10-spin filters (Amicon, Beverly, MA).

Growth of antigen-specific CTL. DC cultures were grown in T-75 tissue culture flasks to a density of 5×10^6 cells/flask in the presence or absence of cytokine or huCD40LT for the last 18 h of culture. On day 9 of culture at least 30 min prior to adding immunomodulatory agents, DC were pulsed with 10 $\mu\text{g/ml}$ of either Flu-M1⁵⁸⁻⁶⁶ or MART-1²⁷⁻³⁵ peptide. On day 10 DC cultures were irradiated (6000 rad) and medium was removed and replaced with AIM-V medium containing 5×10^7 PBMC responder cells. All CTL cultures received IL-7 (10 ng/ml) at the establishment of culture and IL-7 (10 ng/ml) and IL-2 (25 IU/ml) on day 5 of culture. CTL were grown for 10 days prior to assay or they were restimulated with peptide-pulsed adherent PBMC on day 10 and harvested for assay on day 17. Blocking of IL-15 and IL-12 was performed by addition of neutralizing monoclonal antibodies, M112 (10 $\mu\text{g/ml}$) (Genzyme, Cambridge, MA) and C8.6 (15 $\mu\text{g/ml}$) (Pharmingen, San Diego, CA), respectively, at the establishment of antigen-specific CTL cultures. Isotype control was the IgG₁ 107.3 antibody (Pharmingen).

Cytotoxicity assay. After 9 or 17 days in culture, graded doses of effectors were plated in 96-well round-bottom plates with 5000 T2 target cells incubated overnight with either antigen-specific or an irrelevant control peptide and labeled with ^{51}Cr (Amersham, Arlington Heights, IL). After 4 h, supernatants were harvested using a harvesting frame (Skatron) and released chromium-labeled protein was measured using a gamma counter (Packard Instruments). Percentage of antigen-specific lysis was determined by subtracting the percentage of lysis with irrelevant HLA-A2 restricted peptide-pulsed T2 targets from the percentage of lysis with antigen peptide-pulsed T2 targets.

ELISPOT assay. IFN- γ ELISPOT assays were performed using a modification of a protocol established by Fujihashi *et al.* (47). On day 1 mouse anti-human IFN- γ capture antibody NIB42 (10 $\mu\text{g/ml}$) (Pharmingen) was aliquoted into MAHA S4510

plates (Millipore Corporation, NY) and incubated for 18 h at room temperature. On day 2 the supernatant was removed, and blocking buffer (RPMI with 10% fetal bovine sera) (Omega Scientific) was added and allowed to incubate at 37°C for 1 h. Blocking buffer was removed and replaced with graded doses of effector cells and 10⁵ T2 cells pulsed overnight at 37°C with either antigen-specific or irrelevant peptide. On day 3 the plates were washed three times with PBS and three times with PBS containing 0.05% Tween 20 followed by overnight incubation at 4°C with a biotin-labeled anti-human IFN- γ detection antibody 4S.B3 (2.5 μ g/ml) (Pharmingen). On day 4 the biotin-conjugated antibody was removed and plates were washed with TBS followed by a 1-h incubation at 37°C with streptavidin-alkaline phosphatase (1:2000 in TBS) (Gibco, Gaithersburg, MD). Plates were washed three times with TBS. BCIP/NBT color solution (Kirkegaard-Parry Laboratories, Gaithersburg, MD) was added and allowed to incubate for 15–25 min. IFN- γ -producing cells/well were enumerated using a SZH stereo zoom microscope as well as an automated ELISPOT reader (Scientific Products, Los Angeles, CA).

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Conversion of tumor-specific CD4⁺ T-cell tolerance to T-cell priming through *in vivo* ligation of CD40

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Tumor antigen-specific T-cell tolerance limits the efficacy of therapeutic cancer vaccines. Antigen-presenting cells mediate the induction of T-cell tolerance to self-antigens. We therefore assessed the fate of tumor-specific CD4⁺ T cells in tumor-bearing recipients after *in vivo* activation of antigen-presenting cells with antibodies against CD40. Such treatment not only preserved the responsiveness of this population, but resulted in their endogenous activation. Established tumors regressed in vaccinated mice treated with antibody against CD40 at a time when no response was achieved with vaccination alone. These results indicate that modulation of antigen-presenting cells may be a useful strategy for enhancing responsiveness to immunization.

The foundation of cancer immunotherapy rests on the ability of the adaptive immune response to specifically recognize and reject cancer cells in the tumor-bearing host. Although multiple effector mechanisms can be recruited to participate in tumor rejection, it is the T-cell arm of the response that achieves tumor specificity, and CD4⁺ T cells in particular that orchestrate the activities of both antigen-specific as well as nonspecific elements of the tumoricidal response¹⁻⁵.

In a T-cell receptor (TCR) transgenic model, CD4⁺ T cells specific for a model tumor antigen are rendered tolerant early in the course of tumor progression⁶. This tolerance is antigen-specific, and occurs when other elements of the T-cell repertoire function normally. Given the central role of CD4⁺ T cells in the anti-tumor immune response, defining the mechanism(s) responsible for the induction of CD4⁺ T-cell tolerance to tumor antigens is necessary for the successful development of therapeutic cancer vaccines⁷.

Studies of T-cell tolerance to peripheral self-antigens have demonstrated that bone marrow-derived antigen-presenting cells (APCs) are involved in the induction of tolerance to antigens expressed by non-hematopoietic tissues⁸. Given that host APCs are also required for initiating productive T-cell responses, the state of activation and/or differentiation of the APC may be the requisite determinant of whether T cells are primed or rendered tolerant.

The engagement of CD40 on APCs by its ligand CD154 on CD4⁺ T cells is an important event during APC activation^{9,10}. CD40 ligation on dendritic cells results in the upregulation of costimulatory molecules and the secretion of inflammatory cytokines that are central to the initiation of cell-mediated immune responses. We therefore sought to determine whether the *in vivo* activation of APCs using CD40-activating antibodies could provide the signal(s) needed to induce activation rather than tolerance of CD4⁺ T cells. Using a lung metastasis model of a murine renal-cell carcinoma, we found that CD40 ligation not only preserved the responsiveness of tumor-specific CD4⁺ T cells to immu-

nization, but also led to their endogenous activation in the absence of vaccination. Furthermore, immunization of tumor-bearing mice previously treated with antibody against CD40 resulted in substantial tumor rejection, which was not found after vaccination alone. These results indicate that modulating host responsiveness to immunization through APC activation may be a useful strategy to enhance the efficacy of tumor vaccines.

CD40 ligation preserves responsiveness to vaccination

CD4⁺ T cells specific for an antigen expressed exclusively by a B-cell lymphoma are rendered tolerant during the course of tumor progression⁶. Similar findings have been obtained in a lung metastasis model of renal cell carcinoma (E.M.S. *et al.*, manuscript in preparation). In both systems, tumor antigen-specific transgenic CD4⁺ T cells transferred into a tumor-bearing host undergo a transient clonal expansion and have a phenotype associated with antigen recognition. However, functional analysis demonstrates that these cells have a diminished response to peptide antigen *in vitro*, and are unable to be primed *in vivo*.

We determined whether *in vivo* ligation of CD40 could prevent the development of unresponsiveness of TCR transgenic CD4⁺ T cells specific for an MHC class II epitope of influenza hemagglutinin¹¹ (HA) after their transfer into mice with established pulmonary metastases of a renal cell carcinoma expressing HA (RencaHA). Immunization of non tumor-bearing mice with a recombinant vaccinia encoding HA (vac-HA) resulted in a clonal expansion of HA-specific T cells (Fig. 1). The percentage of clonotype-positive T cells in the spleen of a vac-HA primed mouse 6 days after immunization was almost 400% greater than the frequency in an unimmunized mouse (1.42% and 0.37%, respectively; Fig. 1a). However, the response to vac-HA immunization was substantially impaired in a RencaHA-bearing mouse, resulting in a minimal increase in the percentage of clonotype-positive T cells relative to an unimmunized tumor-bearing mouse (0.71% and 0.58%, respectively). In contrast, treatment of tumor bearing

Table 1 Effect of xenogeneic immunoglobulin on T cell responsiveness

| Group | IL-2 | IFN- γ |
|----------------------------|---|---|
| | Mean pg/ml per 100 clonotype ⁺ T cells | Mean pg/ml per 100 clonotype ⁺ T cells |
| No tumor | 5.9 \pm 2.3 | 137 \pm 80 |
| No tumor + rat IgG control | 8 \pm 3.2 | 163 \pm 47 |
| Renca HA | 1.9 \pm 0.2 ^a | 41 \pm 3.0 ^a |
| Renca HA + rat IgG control | 2.4 \pm 0.6 ^a | 54 \pm 6.0 ^a |
| Renca HA + anti-CD40 | 7.4 \pm 0.4 | 197 \pm 5.0 |

RencaHA-bearing mice or tumor-free mice received anti-HA TCR⁺ transgenic T cells and were treated with either the agonist CD40 antibody FGK45 or a similar amount of polyclonal rat IgG control antibody. On day +15 after T cell-transfer, all the mice were immunized with vac-HA and were killed 6 d later; production of IL-2 and IFN- γ was determined by ELISA. Data represent mean \pm s.e.m. of triplicate cultures from three mice in each group, and are expressed as the amount of cytokine produced per 100 clonotype-positive T cells. ^aNot statistically significant.

mice with activating antibody against CD40 (FGK45, ref. 12) resulted in preservation of the response to vac-HA, as demonstrated by a clonal expansion that was similar to that in a tumor-free mouse (1.47% versus 1.42%, respectively). Statistical comparison of the response to immunization (Fig. 1b) verified that tumor-bearing mice were significantly impaired in their response to vac-HA priming compared with tumor-free mice ($P = 0.003$). Furthermore, this response was preserved in RencaHA-bearing mice treated with activating antibodies against CD40 ($P = 0.007$).

CD40 ligation results in preservation of CD4⁺ T-cell function

T cells from vaccinated tumor-free mice fulfill several functional criteria indicative of effective T-cell priming—*in vivo* clonal expansion (Fig. 1b, stippled bar), increased IL-2 production (Fig. 2a, stippled bar) and differentiation into effector cells capable of producing gamma interferon (IFN- γ) after *in vitro* stimulation with HA peptide (Fig. 2b, stippled bar). Most of the increase in the antigen-specific proliferative response after immunization is attributable to the increased numbers of antigen-specific T cells that are generated¹³, such that on a 'per-cell' basis, HA-specific proliferation does not reflect priming (Fig. 2c).

Analysis of HA-specific cytokine release from the splenocytes of RencaHA-bearing mice showed significant impairment in their capacity to produce IL-2 ($P = 0.005$) and IFN- γ ($P < 0.001$; Fig. 2a and b). This unresponsiveness was antigen-specific, as demonstrated by the equivalent responses to vaccinia antigens *in vitro* of T cells obtained from these same vac-HA primed, RencaHA-bearing mice and non-tumor-bearing mice (data not shown). In contrast, treatment of RencaHA-bearing mice with

antibody against CD40 preserved the IL-2 ($P < 0.001$) and IFN- γ ($P < 0.001$) response to peptide antigen. Although T cells from tumor-bearing mice treated with antibody against CD40 proliferated more after HA peptide treatment *in vitro* than those from untreated mice, this difference was not statistically significant (Fig. 2c). Overall, the *in vivo* ligation of CD40 in mice with metastatic RencaHA resulted in a degree of clonal expansion and production of IL-2 and IFN- γ that was similar to that in immunized mice without tumors.

To insure that the observed effects of antibody treatment were a consequence of CD40 ligation rather than a result of the host response to xenogeneic protein (FGK45 is a rat IgG), we compared the effect of antibody against CD40 with that of a control antibody (polyclonal rat IgG) after injection into tumor-bearing mice (Table 1). Whereas treatment of tumor-bearing mice with antibody against CD40 preserved the ability of clonotype-positive T cells to produce IL-2 and IFN- γ in response to vac-HA (similar to immunized tumor-free mice), T cells from tumor-bearing mice treated with control antibody (and mice not receiving antibody) remained impaired in these responses.

Endogenous priming of tumor-specific T cells by CD40 ligation

If ligation of CD40 leads to the activation of APCs that have processed tumor antigen, one might expect this to result in the priming of tumor-specific T cells, even in the absence of immunization. To address this, we assessed the function of clonotype-positive CD4⁺ T cells isolated from the draining lymph nodes (peritracheal, peribronchial and mediastinal) of mice with pulmonary metastases of RencaHA. As in other tumor systems,

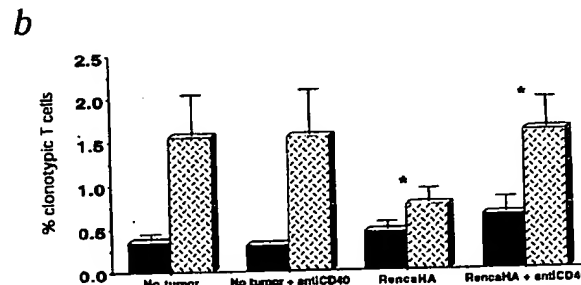
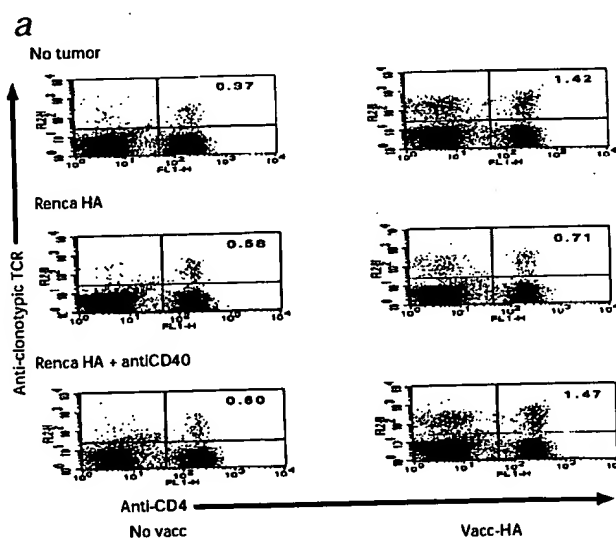


Fig. 1 Effect of CD40 ligation on the responsiveness of RencaHA bearing mice to vaccination. Tumor-bearing mice or tumor-free mice received anti-HA TCR⁺ transgenic T cells and were treated with the agonist CD40 antibody FGK45 (antiCD40). Mice were immunized with vacc-HA 15 d after T-cell transfer and were killed for analysis 6 d later. T cells were analyzed by two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype. **a**, Two-color FACS analysis of splenocytes from non-tumor-bearing and RencaHA-bearing mice. T cells from unimmunized mice (No vac) or immunized mice (vac-HA) were analyzed. Upper right quadrant numbers, percentage of double-positive T cells. **b**, T cells from unimmunized mice (■) and vac-HA-immunized mice (▨) were analyzed by flow cytometry. Data represent mean \pm s.e.m. of the percentage of T cells co-expressing CD4 and the clonotypic TCR for six mice/group (combined results of two independent experiments with three mice per group per experiment). *, $P = 0.007$ for the difference in clonal expansion in response to vac-HA between RencaHA and RencaHA/anti-CD40.

Fig. 2 Effect of *in vivo* ligation of CD40 on the functional responses of antigen-specific CD4⁺T cells. T cells from the mice in Fig. 1 were isolated and assessed for their functional response to HA peptide *in vitro*. **a** and **b**, Production of IL-2 (**a**) and IFN- γ (**b**) in response to *in vitro* stimulation with HA₁₁₀₋₁₂₀ peptide. Purified T cells from unimmunized (■) or vac-HA immunized (▨) mice were stimulated with HA peptide for 48 h; supernatants were collected and assayed for IL-2 or IFN- γ by ELISA. Data represent mean \pm s.e.m. of triplicate cultures from six mice in each group, and are expressed as the amount of cytokine produced per 100 clonotype-positive T cells. *, $P < 0.001$. **c**, Proliferative response to stimulation with HA peptide. T cells from unimmunized (■) or vac-HA immunized (▨) mice were mixed with fresh splenocytes and HA peptide. Data represent mean \pm s.e.m. of the cpm per 100 clonotype-positive T cells per well.

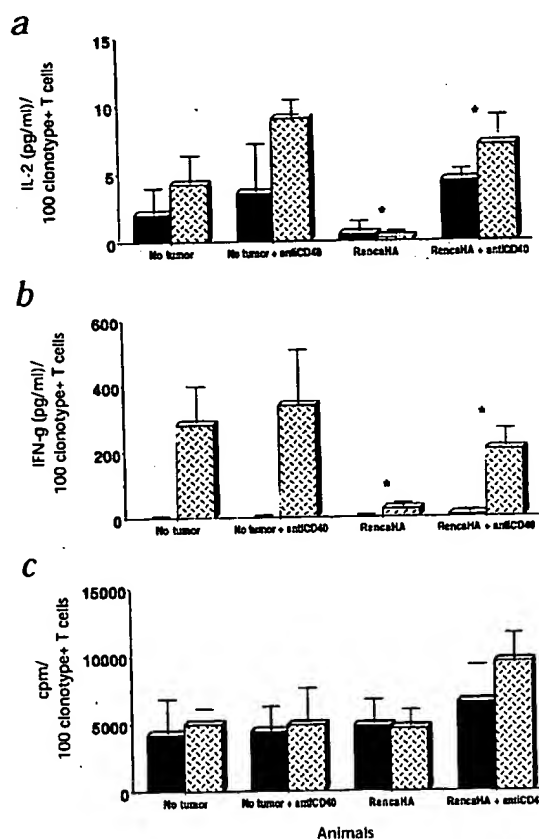
there was an expansion of the population of clonotype-positive T cells in RencaHA-bearing mice relative to that in tumor-free mice (Fig. 3a). This expansion was enhanced in RencaHA-bearing mice treated with activating antibodies against CD40, although this difference was not statistically significant. Similarly, there was a trend towards increased proliferation in response to HA peptide *in vitro* in T cells isolated from tumor-bearing mice treated with antibody against CD40 (Fig. 3b). However, an analysis of cytokine release in response to HA peptide showed that clonotype-positive T cells from tumor-bearing mice treated with antibody against CD40 secreted more IL-2 than those from either naive or untreated tumor-bearing mice ($P = 0.036$; Fig. 3c). Furthermore, these cells had acquired the capacity to produce IFN- γ even in the absence of *in vivo* priming with vac-HA ($P = 0.01$; Fig. 3d). Whereas the IFN- γ production on a 'per-cell' basis was significantly lower than that in response to vac-HA priming (18 and 200 pg/ml per 100 clonotype-positive cells, respectively), the capacity of tumor-specific T cells to produce this cytokine is indicative of endogenous differentiation into functional effector cells.

Anti-tumor effect of treatment with antibody against CD40

Given that CD40 ligation preserved the responsiveness of RencaHA-bearing mice to vac-HA priming, we determined whether treatment with antibody against CD40 alone or in combination with vaccination had any anti-tumor effect in this pulmonary metastasis model of renal cell carcinoma. The lung of an untreated RencaHA-bearing mouse contained many malignant nodules 3 weeks after T-cell transfer (Fig. 4). Vac-HA given 6 days before the mouse was killed did not have any demonstrable anti-tumor effect (Fig. 4, Vac-HA), as expected, given the T-cell unresponsiveness demonstrated above. Further analysis of six mice per group demonstrated that all unimmunized or vac-HA-immunized mice examined at this time had more than 20 pulmonary metastases (data not shown). Similarly, the lungs of mice receiving control (polyclonal rat IgG) antibody, either alone or in combination with vac-HA, had a tumor burden that was indistinguishable from that of untreated mice.

In a group of mice treated with antibody against CD40 alone (given on days -1 and +1), four of the six mice had more than 20 large tumor nodules at the time of analysis. One mouse that was initially thought not to have tumor by macroscopic examination of the lung was subsequently found to have malignant nodules by histologic examination (Fig. 4, Anti-CD40). The other mouse was tumor-free, as ascertained by careful histologic examination. This modest anti-tumor effect of treatment with antibody against CD40 alone may be a consequence of the endogenous activation of T cells (Fig. 3).

Whereas vac-HA immunization given 15 days after T-cell trans-



fer led to no substantial anti-tumor response, identical immunization of RencaHA-bearing mice that were previously treated with CD40-activating antibodies resulted in a definite anti-tumor effect. Of the six mice treated in this way, two had neither macroscopic nor microscopic evidence of pulmonary metastases at the time of analysis (day +21). Furthermore, *in vitro* culture of lung explants from these mice failed to show any tumor growth (data not shown). The remaining four mice had fewer than ten pulmonary nodules per mouse (a tumor burden equivalent to that in untreated mice 1 week after the transfer of T cells), indicative of a substantial delay in tumor growth. A representative photograph of the lung of one of these mice is shown in Fig. 4 (Anti-CD40/Vac-HA). The modest cellular infiltrate in the pulmonary

Table 2 Comparison of IL-4 and IFN- γ responses in tumor-bearing mice and peptide-treated mice.

| Group | IL-4 Mean pg/ml per 100 clonotype ⁺ T cells | IFN- γ Mean pg/ml per 100 clonotype ⁺ T cells |
|------------------------|--|---|
| Renca HA | 0 | 24 \pm 5 |
| Renca HA + anti-CD40 | 0.15 \pm 0.15 | 115 \pm 5 |
| HA peptide | 0.85 \pm 0.05* | 32 \pm 1 |
| HA peptide + Anti-CD40 | 2.3 \pm 0.8* | 40 \pm 9* |

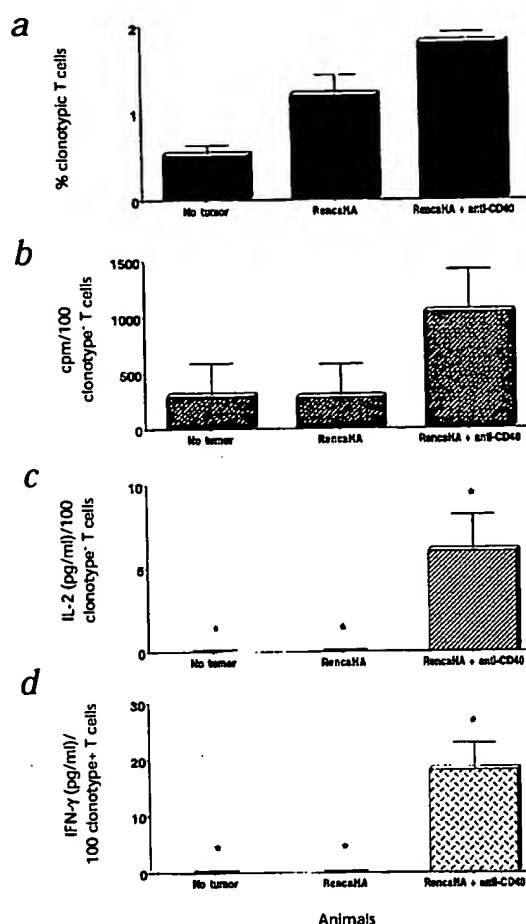
Tumor-bearing mice or tumor-free mice received anti-HA TCR⁺ transgenic T cells and were treated with the agonist CD40 antibody FGK45. Then, 2 d after T-cell transfer, the tumor-free mice were given a tolerogenic dose of HA peptide (275 μ g). All the mice were immunized with vac-HA on day +9 after T-cell transfer and were killed 6 d later. Purified T cells were stimulated with HA peptide *in vitro* for 48 h and then supernatants were assayed for IL-4 and IFN- γ by ELISA. Data represent mean \pm s.e.m. of triplicate cultures from three mice in each group, and are expressed as the amount of cytokine produced per 100 clonotype-positive T cells. * $P = 0.006$, compared with RencaHA.

Fig. 3 Analysis of HA-specific T cells isolated from regional lymph nodes. RencaHA-bearing mice or tumor-free mice received anti-HA TCR⁺ transgenic T cells and were treated with antibody against CD40. On day +21, mice were killed and the thoracic lymph nodes from three mice per group were collected and pooled for analysis. **a**, Lymph node cells were analyzed by two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype. Data represent mean \pm s.e.m. of the percentage of double-positive T cells from two independent experiments. **b**, Proliferative response to HA peptide. Data represent the mean \pm s.e.m. cpm per 100 clonotype-positive T cells per well from two independent experiments. **c** and **d**, Purified T cells were stimulated with HA peptide for 48 h; supernatants were collected and assayed for IL-2 (**c**) or IFN- γ (**d**) by ELISA. Data represent mean \pm s.e.m. of triplicate cultures from three mice in each group, and are expressed as the amount of cytokine produced per 100 clonotype-positive T cells. *, $P = 0.036$, IL-2; *, $P = 0.01$, IFN- γ .

parenchyma of mice receiving antibody against CD40 and vac-HA was somewhat greater than that in the other groups, although the importance of this is uncertain.

CD40 ligation prevents peptide induced CD4⁺ T-cell anergy

As the Renca tumor used in our system, like many tumors of epithelial origin¹⁴⁻²⁰, expresses CD40 and could thereby be recognized directly by FGK45, we sought to determine whether antibody against CD40 could prevent T-cell unresponsiveness in another well-characterized *in vivo* model of T-cell anergy: peptide-induced T-cell anergy^{21,22}. Intravenous administration of HA peptide induced T-cell unresponsiveness to vac-HA (Fig. 5). Compared with those from mice not given peptide, clonotype-positive T-cell populations from mice receiving peptide failed to expand *in vivo* ($P < 0.001$), and were defective in their ability to produce IL-2 ($P = 0.001$) and IFN- γ ($P < 0.001$). In contrast, treatment with antibody against CD40 preserved the response to vac-HA in mice that received intravenous HA peptide. The clonal expansion of HA-specific transgenic T cells was significantly higher in peptide-treated mice given antibody against CD40 than in those receiving peptide alone ($P = 0.015$). Furthermore, treatment with antibody against CD40 prevented the loss of IL-2 production by clonotype-positive T cells in peptide-treated mice ($P < 0.001$). Treatment with antibody against CD40 alone seemed to enhance IL-2 production by T cells encountering an otherwise tolerogenic form of antigen *in vivo*, even in the ab-



sence of immunization with vac-HA.

In contrast to what was seen with tumor-induced T-cell tolerance, CD40 ligation in mice receiving HA peptide failed to preserve the ability of HA-specific CD4⁺ T cells to differentiate into IFN- γ -producing effector cells in response to vac-HA priming (Figs. 2b and 5c). Given that these cells were responsive by other parameters (such as clonal expansion and IL-2 production), we looked for evidence of differentiation along another effector pathway: that is, the production of IL-4. Indeed, the peptide-induced tolerance model (but not the tumor tolerance model) seems to favor the differentiation of clonotype-positive T cells into IL-4-producing cells (Table 2). After treatment with antibody against CD40, the effector response to vac-HA priming is manifest as enhanced IL-4 production. Therefore, CD40 ligation preserves T-cell responsiveness in the peptide tolerance model, but the response is polarized towards the production of a prototypic Th-2 cytokine, which probably accounts for the failure of these cells to make IFN- γ .

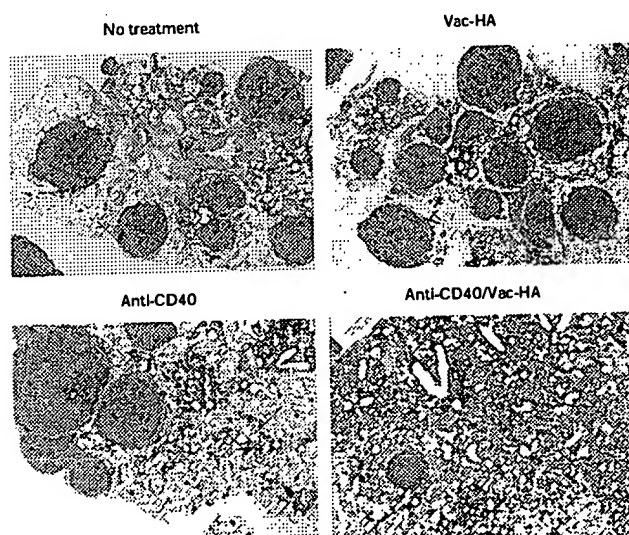


Fig. 4 Effect of CD40 ligation on the anti-tumor response to vaccination. Tumor-bearing mice received anti-HA TCR⁺ transgenic T cells and were treated with the agonist CD40 antibody FGK45. Mice were immunized with vac-HA on day +15 after the adoptive transfer of clonotypic T cells and were killed for analysis 6 d later. Lung samples are from an untreated tumor-bearing mouse (No Treatment), a mouse immunized with vac-HA alone (vac-HA), a mouse treated with CD40 alone (Anti CD40) and a mouse treated with combination therapy (Anti CD40/Vac-HA).

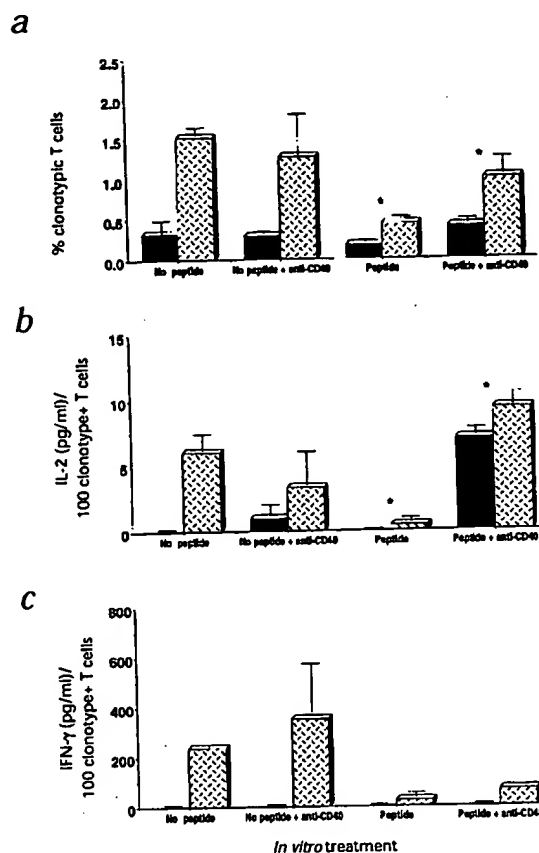
Fig. 5 Effect of CD40 ligation on the response to intravenous HA peptide. Mice that received anti-HA TCR⁺ transgenic T cells either were or were not treated with antibody against CD40 (FGK45) before receiving a tolerogenic dose of HA peptide (275 μ g). Vac-HA was given 15 d after T-cell transfers, and analysis was done 6 d later. **a**, T cells isolated from unimmunized (■) and vac-HA immunized mice (▨) analyzed by two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype. Data represent the mean \pm s.e.m. of the percentage of T cells co-expressing CD4 and the clonotypic TCR for three mice/group. *, $P = 0.015$. **b** and **c**, Purified T cells were stimulated with HA peptide for 48 h, then supernatants were collected and assayed for IL-2 (**b**) or IFN- γ (**c**) by ELISA. Data represent mean \pm s.e.m. of triplicate cultures from three to four mice in each group. Data are expressed as the amount of cytokine produced per 100 clonotype-positive T cells per well. *, $P < .001$, IL-2.

Discussion

Much attention has been given to the observation that tumor cells, typically being the transformed counterparts of 'non-professional' antigen-presenting cells, lack the capacity to express T-cell co-stimulatory molecules. In the absence of adequate co-stimulation, the direct encounter of T cells with tumor cells has been proposed as the basis for the development of tumor antigen-specific T-cell tolerance. However, the requirement for a direct T cell-tumor interaction in the development of tumor-specific CD4⁺ T-cell tolerance is problematic. Most tumors of nonhematopoietic origin do not express MHC class II molecules (although expression can often be induced in the presence of IFN- γ and tumor necrosis factor (TNF- α)). Moreover, naive T cells circulate mainly between the blood and the secondary lymphoid compartments, only entering the extra-lymphoid tissue spaces after activation and acquisition of effector function. Although solid tumors do often metastasize through the lymphatics (an event that may have important immunologic consequences), in our model of CD4⁺ T-cell tolerance to renal cell carcinoma, we have not identified lymphatic tumor, indicating that tolerance does not depend on this event.

Alternatively, the induction of tumor-specific CD4⁺ tolerance may involve the presentation of tumor antigen by host APCs. APCs are indispensable in establishing peripheral T-cell tolerance to normal self-antigens. Bone marrow-derived APCs can capture and present peripheral tissue-specific antigens to naive CD8⁺ T cells, leading to their deletion^{23,24}. There is a similar requirement for the processing of parenchymal self antigen by host APCs in the induction of CD4⁺ T-cell tolerance²⁵.

In the immune response to tumors, one plausible scenario therefore involves the capture of tumor antigen at the tumor site by host APCs that then migrate to the T-cell zone of secondary lymphoid organs for presentation to tumor-specific T cells. Consistent with this is the observation that anti-HA CD4⁺ T cells isolated from the draining lymph nodes and spleen of RencaHA-bearing mice undergo an initial clonal expansion accompanied by an increase in size (forward light scatter) and loss of naive phenotype, compared with that of HA-specific T cells



from tumor-free mice and mice with Renca wild-type tumors (data not shown). These changes are consistent with transgenic T cells encountering antigen on cells capable of providing some degree of co-stimulation. Despite these changes, these same T cells are functionally impaired, as shown by their inability to proliferate *in vivo* in response to vaccination, as well as their decreased capacity to produce cytokines when re-stimulated with the nominal peptide antigen *in vitro* (Figs. 1b and 2).

Given that bone marrow-derived APCs are also essential for T-cell priming, these observations lead to the hypothesis that the differentiation and/or activation state of the APC is the central determinant of T-cell priming versus tolerance²⁶. In their immature state, APCs such as dendritic cells have relatively low levels of MHC, co-stimulatory molecules and other adhesion molecules that participate in T-cell priming²⁷. Nonetheless, immature dendritic cells can efficiently capture peptide fragments from apoptotic cells²⁸ and present peptide antigens derived from this material in the T-cell zones of lymphoid tissues²⁹. In the absence of APC acti-

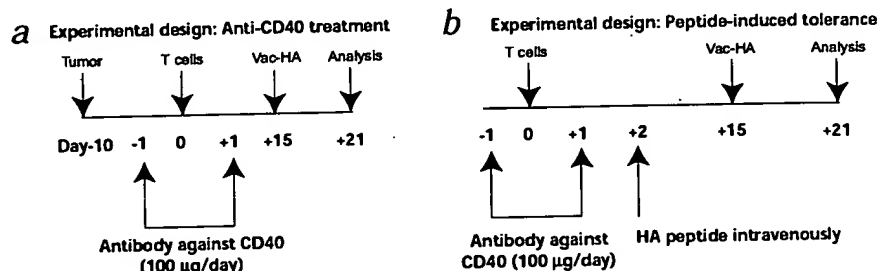


Fig. 6 Experimental protocols. Treatment and analysis protocols used for experiments in Figs. 1 and 2 (**a**) and Fig. 5 (**b**).

vation, this process has been proposed to mediate the induction and maintenance of peripheral tolerance to self antigens⁸. This pathway may well typify how the immune system normally encounters tumor antigens³⁰.

In contrast, the ability of the innate immune response to promote T-cell priming and cell-mediated immunity has been attributed to the production of factors that induce APCs to upregulate the expression of T-cell co-stimulatory molecules and to produce inflammatory cytokines³¹⁻³³. This forms the basis of the efficacy of adjuvants often used in vaccine formulations. Therefore, strategies aimed at providing signal(s) that lead to effective APC activation *in vivo* have the potential to convert a T-cell encounter with antigen/APC from a tolerizing event into a priming event. Indeed, as demonstrated here, *in vivo* activation of APCs achieved through triggering of CD40 not only preserved the responsiveness of tumor-specific CD4⁺ T cells to vaccination in tumor-bearing mice (Fig. 2) but also resulted in their endogenous activation rather than tolerance (Fig. 3). Similarly, *in vivo* ligation of CD40 on APCs resulted in the priming of CD4⁺ T cells in response to an otherwise tolerogenic dose of peptide injected intravenously (Fig. 5).

The importance of APC activation through CD40 engagement has been recently emphasized by the demonstration that a principal component of the T-helper cell function that is required for priming MHC class I-restricted CTLs is mediated through the engagement of CD40 on APCs by its ligand on CD4⁺ T cells. The resulting activation of APCs is sufficient to drive naive CD8⁺ T cells to become fully activated effector cells³⁴⁻³⁶.

However, a model in which CD4⁺ T cells alone are sufficient to activate APCs does not account for how CD4⁺ T-cell responses can possibly be regulated. Specifically, if all that is required for the 'licensing' of APCs to activate T cells (including CD4⁺ T cells themselves) is the cognate interaction between an antigen-specific T-helper cell and an APC presenting its antigen, then the outcome of all such encounters would be priming, including that of self-reactive T cells. Although there is definite evidence that the provision of T-helper cell function can indeed convert MHC class I-restricted CTL tolerance to T-cell priming^{37,38}, there is equally compelling evidence that CD4⁺ T cells can be rendered tolerant *in vivo*^{8,22,25,39-43}, as was seen here.

Perhaps after an initial encounter with antigen, naive CD4⁺ T cells are often not capable of providing sufficient signals to activate or 'license' an otherwise immature APC. The outcome of such an encounter would be CD4⁺ T-cell tolerance. The fact that CD4⁺ T-cell tolerance is seen even at the experimentally high CD4⁺ T-cell precursor frequencies used in this and other studies further emphasizes the point that the availability of antigen-specific T helper-cell action alone cannot be the sole determinant regulating the induction of cell-mediated immunity.

Instead, it is likely that the pathway leading to APC activation is normally initiated as a consequence of the innate immune response to a pathogen and/or as a direct response of APCs to infection³⁴. One of the most salient changes in the phenotype of activated versus immature dendritic cells is the increased expression of CD40 (ref. 27), as occurs after exposure to bacterial lipopolysaccharide⁴⁴. Perhaps this renders the APC more receptive to T-helper cell function. Given the profound impairment of the ability to prime T-cell responses in mice with the targeted disruption of CD40 or CD40 ligand, it is likely that CD4⁺ T cells are important in sustaining and perhaps amplifying APC activation after it is initiated. Anergic CD4⁺ T-cells are very deficient in their ability to upregulate CD40 ligand⁴⁵. We are now assessing

the ability of HA-specific CD4⁺ T cells from RencaHA-bearing mice to express CD40 ligand.

From a therapeutic perspective, it seems that this 'insufficient cross-talk' between tumor-specific CD4⁺ T cells and host APCs in tumor-bearing mice is either corrected (if defective) or at least augmented after the exogenous triggering of CD40 with antibodies. The activation of APCs using this strategy not only converted T-cell tolerance to T-cell activation, but also preserved the responsiveness of tumor bearing mice to vaccination. Therefore, the advances made in vaccination against infectious pathogens using CD40-activating antibodies as 'adjuvants' (refs. 46,47) has now been extended to the field of tumor vaccines, indicating that modulation of APCs may be useful in enhancing the efficacy of this therapeutic modality.

Methods

Mice. Male BALB/c mice 6-8 weeks old were obtained from the National Institutes of Health (Frederick, Maryland). TCR transgenic mice expressing an $\alpha\beta$ T-cell receptor specific for amino acids 110-120 from influenza hemagglutinin presented by I-E* were a gift from H. von Boehmer¹¹. These mice were crossed to a BALB/c background for more than ten generations. The transgenic mice used in these experiments were heterozygous for the transgene. All experiments involving the use of mice were in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Tumor cells. Renal cell carcinoma cells (Renca) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). Cells were cultured *in vitro* in RPMI 1640 media, supplemented with 10% FCS, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 50 mM β -mercaptoethanol (complete media), and were grown as an adherent population at 37 °C, 5% CO₂. RencaHA was generated by calcium phosphate-mediated plasmid transfection with the construct pHA, which encodes the HA molecule of the influenza virus A/PR/8/34 (H1N1), as reported⁴⁸. RencaHAnco was selected and grown in complete media supplemented with the neomycin analog G418 (400 μ g/ml).

Adoptive transfer. Single-cell suspensions were made from peripheral lymph nodes and spleen collected from TCR transgenic donors. The percentage of lymphocytes double-positive for CD4 and the clonotypic TCR was determined by flow cytometry. Cells were washed three times in sterile Hanks balanced salt solution (HBSS), and injected into the tail veins of male BALB/c recipients such that a total of 2.5×10^6 CD4⁺ anti-HA TCR⁺ T cells was transferred to each recipient. RencaHA cells used for *in vivo* tumor challenge were detached from the culture flasks with trypsin (Sigma) and were suspended in complete media. Then, cells were counted and viability was assessed by trypan blue exclusion. If the viability was 100%, tumor cells were washed three times in sterile HBSS, and injected through tail vein in a total volume of 0.2 ml, 1×10^6 tumor cells per mouse.

***In vivo* treatment with activating antibodies against CD40.** The experimental design in Fig. 6a was used in the experiments in Figs. 1 and 2. Pulmonary metastases of RencaHA were established in BALB/c mice by intravenous injection of 1×10^6 tumor cells. After 10 d, transgenic anti-HA CD4⁺ T cells (2.5×10^6) were transferred intravenously into these recipients or into tumor-free mice (day 0). Half the mice in each group received 100 μ g/day of the agonist CD40 antibody FGK45 given intravenously on days -1 and +1. Similarly, a subgroup of mice received 100 μ g of polyclonal rat IgG (Sigma) intravenously on days -1 and +1 after T-cell transfer. On day +15 after T-cell transfer, half the mice in each subgroup were immunized subcutaneously with 1×10^7 plaque-forming units of a recombinant vaccinia virus encoding influenza hemagglutinin (vac-HA). In all experiments, three mice per subgroup were used and mice were analyzed individually. Each mouse was given a unique identification number so that specific determinants of T-cell responsiveness could be correlated within an individual as well as between mice in the same group or between groups. Mice were killed 6 d after immunization (day +21 after T-cell transfer) for analysis.

Assessment of pulmonary metastases. Mice were killed, and after the thoracic cage was opened, the lungs were carefully dissected. Lungs were washed with HBSS and evaluated for the presence of tumor nodules on a scale of 1+ to 3+: Fewer than 10 nodules/lung, 1+; 10–20 nodules/lung, 2+; more than 20 nodules/lung, 3+ or 'significant tumor burden'. Then, one lung was fixed in formalin, paraffin-embedded, and stained with hematoxylin and eosin. RencaHA nodules were explanted from the remaining lung and a single-cell suspension was made by mechanical dissociation and passage through nylon mesh. Explants of RencaHA obtained at different time points during tumor progression demonstrated continued expression of HA, as determined by staining with the antibody against HA, H-18 (data not shown).

Re-isolation of clonotypic T cells after *in vivo* transfer. On the day of analysis, spleen cells were obtained by passing them through nylon mesh and centrifugation on a Ficoll gradient (Ficoll-Paque; Pharmacia). Then, splenocytes were passed through nylon wool to enrich samples for T cells. Optimization of this technique has allowed us to obtain at least 5×10^6 highly purified T cells per spleen, an amount sufficient for our studies.

Flow cytometric analysis. T cells were stained with FITC-conjugated goat anti-mouse CD4 (Caltag, Burlingame, California) and biotinylated rat anti-clonotypic TCR antibody MAb 6.5, followed by PE-conjugated streptavidin (Caltag, Burlingame, California). For this analysis, 50,000 gated events were collected on a FACSCAN (Becton Dickinson, San Jose, California) and analyzed using CellQuest software (Becton Dickinson, San Jose, California). Data represent the mean \pm s.e.m. of the percentage of cells expressing the clonotypic TCR. Background staining of splenocytes or lymph node cells from naive BALB/c mice is usually less than 0.10%.

Antigen-specific proliferation. Purified T cells (4×10^4 cells/well) from the experimental groups were mixed with fresh splenocytes (8×10^4 cells/well) from naive BALB/c mice to which 12.5 μ g/ml of synthetic HA peptide (amino acids 110–120; SFERFEIPKE) was or was not added. The cells were pulsed with 3 H-thymidine (1 mCi/well, Amersham) after 3 d in culture. Cells were collected 18 h later with a Packard Micromate cell harvester. Thymidine incorporation into DNA was measured as counts per minute (cpm) on a Packard Matrix 96 direct beta counter. Data represent as cpm per 100 clonotype-positive T cells.

Cytokine release. T cells purified and plated as described above were cultured with media alone or HA peptide (12.5 μ g/ml) plus fresh BALB/c splenocytes. Then, 48 h later, supernatants were collected and stored at -70°C , then assayed for IL-2, IL-4 and IFN- γ by ELISA (R&D Systems, Minneapolis, Minnesota). Values for T cells cultured in media alone were less than 10% of the values for HA-stimulated T cells. Data represent pg/ml of the specific cytokine per 100 clonotype-positive T cells per well.

Analysis of clonotypic T cells from draining lymph nodes. To assess the fate and function of those clonotype-positive T cells in the regional lymph nodes, the peritracheal, peribronchial and mediastinal lymph nodes were collected from tumor-free mice and from RencaHA-bearing mice. Lymph nodes from three mice per group were pooled, and cell suspensions were made by passing the samples through nylon mesh and centrifugation on a Ficoll gradient. Between 2×10^6 and 3×10^6 lymph node cells were obtained from the pooled samples of tumor-bearing mice, and between 0.5×10^6 and 1×10^6 cells were obtained from tumor-free mice. The phenotypic and functional characteristics of these cells were analyzed as described above (flow cytometric analysis, antigen-specific proliferation and cytokine production).

Intravenous injection of a tolerogenic dose of HA peptide₁₁₀₋₁₂₀. The experimental design in Fig. 6b was used to evaluate the effect of antibody against CD40 treatment in a well-characterized model of peptide-induced tolerance (Fig. 5). Anti-HA/I-E^c TCR⁺ transgenic T cells (2.5×10^6) were transferred into BALB/c mice on day 0. Half the mice received 100 μ g/day of the agonist antibodies against CD40 intravenously 1 d before and 1 d after the transfer of T cells. On day +2, an intravenous injection of 275 μ g purified HA peptide₁₁₀₋₁₂₀ was given to some mice. On day +15 after T-cell transfer, half the mice in each subgroup were immunized subcutaneously

with 1×10^7 plaque-forming units of vac-HA. All the mice were killed for analysis 6 d after immunization (day +21), and T cells from the spleen were obtained. Phenotypic and functional characteristics of these reisolated T cells were evaluated as described above.

***In vivo* priming with vac-HA.** A recombinant vaccinia virus encoding hemagglutinin from the 1934 PR8 strain of influenza was a gift from F. Guarneri. Vac-HA was amplified on Hu-TK⁺ cells in the presence of 25 μ g/ml 5-bromo-2'-deoxyuridine (Sigma). Virus was purified from the cellular lysate by sucrose banding, and titered by plaque assay on B-SC-1 cells. On the days indicated for each particular experimental design, mice were primed by subcutaneous inoculation with 1×10^7 plaque-forming units of recombinant vaccinia encoding HA suspended in 0.1 ml HBSS.

Statistical analyses. Two-way analysis of variance (ANOVA) was used to evaluate the magnitudes of tumor and antibody against CD40-induced effects for clonotypic T-cell expansion, proliferation and cytokine production. To compare the experimental groups in Fig. 3, we used a one-way ANOVA.

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